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Review

The Use of Animal Models in Understanding Human Teratogens

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ABSTRACT The testing of drugs and other chemicals in pregnant animals is required by legislation in a number of countries as a screening procedure for teratogenic potential in the human. The testing procedure involves methodology designed in the 1960s which was based on regimens established in the 1940s for toxicity testing. The requirement that animals are dosed to maternally toxic levels, frequently means that the embryos are exposed to inappropriately high concentrations of the test substance. Positive results in this type of experiment may have no relevence to the human situation where the exposure profile is often quite different, with the human embryo being exposed for prolonged periods to much lower drug concentrations. One way of duplicating the anticipated human exposure is to grow rat embryos in serum containing the drug and/or its metabolites at concentrations determined in the human during early clinical testing. It is proposed that mammalian embryos will respond in a similar manner to a particular concentration of a test substance.

In vitro experiments using isotretinoin and its main metabolite 4-oxo-isotretinoin showed that the metabolite was teratogenic at concentrations which occurred in the human during normal repetitive dosing and hence the metabolite was the likely human teratogen. Similarly, rat embryo culture studies showed that the anticonvulsant drug, valproic acid, was teratogenic at blood concentrations which occurred during normal dosing in the human. Other in vitro studies showed that cadmium is unlikely to be a human teratogen, despite the fact that it is well established as a teratogen in experimental animals in vivo.

It is proposed that embryo culture should be used as an adjunct procedure during teratology testing making use of metabolic and pharmacokinetic data obtained from the human during clinical testing.

Key words: teratogens, in vitro, isotretinoin, valproic acid, cadmium, heparin

INTRODUCTION

Predicting the possible teratogenicity of drugs and other chemicals to the human conceptus remains a surprisingly difficult objective for scientists and regulatory bodies. Animal testing has shown that it more surprising when a chemical is apparently safe than when it is teratogenic. The current regimen daily administration of drugs throughout the organogenic period at almost toxic doses usually pro- some evidence of embryotoxicity or teratogenicity. The difficult task facing drug companies ulatory agencies is to try and relate the pattern and level of exposure in the rat, mouse or rabbit

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to the anticipated human exposure. The highest dose used in experimental animals is usually greatly in excess of the intended human dose reflecting the generally more rapid metabolism of rodents and rabbits. The absence of a suitable formula to compensate for these metabolic differences makes the subsequent risk/benefit calculation a very subjective assessment. Not surprisingly, regulatory bodies are forced by these circumstances to be conservative and the myth is nurtured that most drugs are potentially harmful to the developing conceptus and that drugs should not be taken during pregnancy.

Recent advances in analytical chemistry, particularly the development of high pressure liquid chromatography and gas chromatography mass spectroscopy have provided relatively rapid and inexpensive methods for the determination of the metabolism and pharmacokinetics of drugs. It is proposed that the use of this technology and the subsequent testing of drugs and their metabolites by in vitro whole embryo culture would provide data which would allow a more objective assessment of the standard tests used to determine the possible teratogenicity of chemicals. Four examples are given to illustrate the use of these data and possible problems in interpretation.

ISOTRETINOIN

Isotretinoin (Accutane, 13-cis-retinoic acid) is an analogue of vitamin A and is used to treat severe cystic acne as well as other skin disorders; it is also a known human teratogen. Conventional teratology testing had shown that the drug was teratogenic when administered to rats from gestational days 7 to 15 as a daily oral dose of 150 mg/kg with 50 mg/kg being the no effect dose. In rabbits the teratogenic dose was 10 mg/kg as a daily oral dose from day 7 to day 18 of gestation with 3 mg/kg/ day being the no effect dose (Kamm, 1982). The anticipated human dose was 0.5-1.5 mg/kg/ day. Appropriate labelling and other measures were taken to inform the medical profession and the public that the drug was not to be used if there was any possibility of pregnancy. Despite these warnings there have been hundreds of instances of accidental exposure during early pregnancy and at least 62 babies born with severe congenital malformations. The affected children have a characteristic pattern of abnormality called the 'retinoic acid embryopathy' which includes abnormalities of the face, heart, thymus and brain. The difference between the teratogenic dose in rats and rabbits and the intended dose in humans could have been interpreted as indicating that the drug presented no teratogenic risk to the human. However, consideration of the metabolism and pharmacokinetics of the drug, together with appropriate embryo culture studies, would have correctly predicted the teratogenicity of the drug for the human.

In the human the half-life of an orally administered 1 mg/kg dose is about 17 hours (Colburn et al., 1983) while the half life for a 10 mg/kg oral dose in the mouse is about 19 minutes (Kalin et al., 1982). The rate of metabolism largely determines the blood levels and hence the dose needed to produce a critical blood level of the drug and/or metabolites for a period long enough to damage the embryo. Normal repetitive dosing with isotretinoin in the human (40 mg, twice a day for 16-20 weeks), which has been associated with teratogenesis, results in mean trough blood concentrations of 132 to 186 ng/ml for isotretinoin and 610 to 791 ng/ml for the main metabolite, 4-oxo-isotretinoin (Brazzell et al., 1983).

When rat embryos were cultured for 48 hours in serum containing either isotretinoin or 4-oxo-isotretinoin it was found that both chemicals were teratogenic at a concentration of 500 ng/ml with 250 ng/ml being the no effect concentration (Webster et al., 1986). Both substances caused the same

pattern of abnormality in the cultured embryos, namely underdevelopment of the visceral arches, particularly the second visceral arch. This is significant since the second visceral arch forms most of the external ear and external ear defects are characteristic of the retinoic acid embryopathy in the human (Lammer et al., 1985). Deficits in the cranial neural crest contribution to the visceral arches, heart and thymus explain most of the malformations seen in the human. Since the metabolite and parent compound induce the same malformations at the same concentrations in embryo culture it is likely that the higher level of the metabolite in the human is largely responsible for the teratogenic effect.

Hence by knowing the metabolism of the drug in the human it was possible to test both the parent compound and the main metabolite for teratogenicity. The pharmacokinetic information indicated that the human embryo would be exposed to relatively constant concentrations of both compounds for extended periods with the metabolite present at four times the concentration of the parent compound. By exposing rat embryos during their organogenic phase of development to various concentrations of these compounds it was possible to show that the metabolite was present in sufficient concentration in human patients to cause abnormal development; the additional presence of the parent compound, at a much lower concentration, simply increased the teratogenic risk.

VALPROIC ACID

The anti-convulsant drug valproic acid was suggested to be a human teratogen by a retrospective study of data from the Rhone-Alps birth defects surveillance system in France (Robert and Guibaud, 1982). Out of 72 infants with lumbosacral neural tube defects nine were born to epileptic mothers who had taken valproic acid during pregnancy. These cases were reported in response to a letter by Gomez (1981) which described a single case of spina bifida in a valproic acid exposed infant. Valproic acid is now established as a human teratogen causing an increased risk of spina bifida and possibly other malformations (review, Lammer et al., 1987).

Conventional animal testing had shown that the drug was teratogenic in rats and mice at about 600 mg/kg/day (oral) and in the rabbit at between 250-400 mg/kg/day (oral) (Whittle, 1976). The anticipated human dose was 15-60 mg/kg/day. Once again there are large differences in the rate of metabolism between humans and experimental animals. The mean serum half-life of valproic acid is 7-9 hours for adults on chronic therapy (dose 14-37 mg/kg/day) (Pinder et al., 1977) compared with 48 minutes for mice given a 400 mg/kg dose (Nau et al., 1981).

In vitro rat embryo culture studies (Kao et al., 1981) clearly showed that the drug was teratogenic at serum concentrations associated with therapeutic dosing in the human. The therapeutic serum range is 50-150 μg/ml (Browne, 1980). The in vitro tests showed that sodium valproate was teratogenic to early somite rat embryos at serum concentrations of 144 μg/ml producing a high incidence of exencephaly (Kao et al., 1981). At the lower concentration of 86 μg/ml there was no exencephaly but the embryos had irregular neural suture lines with irregular somites. These malformations induced in vitro may be related to the range of human neural tube defects known as spina bifida. is important not to oversimplify these results. Valproic acid is metabolised into a variety of other unds which are present in human serum at detectable levels. These compounds also need ted for their teratogenic potential both alone and in combination. Some of this work has

been done and it appears that valproic acid is the major teratogen and is present in the highest concentrations (Nau, 1986; Lewandowski et al., 1987)

CADMIUM

The third example is the heavy metal and environmental pollutant cadmium (Cd). Cadmium is a teratogen in experimental animals and has been used extensively in studies of the pathogenesis of a range of congenital malformations. Humans are exposed to this metal in almost everything ingested and sometimes inhaled as it is present in cigarette smoke. In this example, studies of metabolism, blood levels and in vitro embryo culture clearly indicate that Cd is not likely to be a teratogen in the human (Webster, 1989).

Cadmium is only teratogenic in rodents following acute, high doses (1-4 mg/kg) usually administered parenterally (Ishizu et al., 1973). Doses of 40 mg/kg administered by gavage have also been reported to be teratogenic (Baranski et al., 1982; Baranski, 1985). In contrast, when Cd is administered in the food, water or air, even at massive doses, it is not teratogenic, although it may cause fetal growth retardation (e.g. Pond and Walker, 1975; Webster, 1978; Prigge, 1978; Ahokas et al., 1980; Machemer and Lorke, 1981; Barenski, 1985). Hence Cd teratogenesis is a feature of exposure procedures which cause sudden and very highly elevated blood Cd levels, such as intraperitoneal injection. Chronic administration does not cause such blood Cd elevations and is not associated with teratogenesis.

A teratogenic dose (6 mg/kg) administered intraperitoneally to pregnant mice produced a peak plasma Cd concentration of 5350 ng/ml with a half life of 30 minutes (Warner et al., 1983; 1984). Using this information 9-day mouse embryos were exposed in whole embryo culture to Cd levels equal to the maternal peak plasma levels for 30 minutes and then transferred to Cd free serum for 48 hours. Seventy-two percent of the treated embryos had neural tube and eye defects similar to those induced by Cd exposure at the same stage in vivo. When the in vitro dose of Cd was reduced to 2700 ng/ml for 30 minutes the malformation rate decreased to 50% (Warner et al., 1983; 1984). In other experiments using rat embryos, Cd was added to the culture medium for the entire duration of the culture period (Record et al., 1982). A concentration of 320 ng/ml killed the embryos while 170 and 80 ng/ml caused severe growth retardation but did not cause malformations, 40 ng/ml caused slight growth retardation. Hence these in vitro studies clearly show that Cd teratogenesis is due to the short-lived peak blood levels associated with administration techniques which cause sudden large elevations in blood Cd. More prolonged exposure to lower concentrations may cause fetal growth retardation, or even death, but not malformations.

These toxic levels used in embryo culture can be compared with the normal blood Cd levels of 0.1-1.2 ng/ml found in pregnant women at the time of delivery (Alessio et al., 1984) and blood Cd levels of 17 ng/ml (range 7-31) in industrially exposed individuals (Roels et al., 1981). It is unlikely that the high blood Cd levels associated with teratogenesis in experimental animals could occur in humans except under the most exceptional circumstances.

HEPARIN

The last example demonstrates some of the problems associated with drug testing in whole embryo culture. Heparin is generally considered the most suitable anticoagulant for use during pregnancy

since it has a high molecular weight (5000-20.000 daltons) and is unable to cross the chorioallantoic placenta (Flessa et al., 1963). There is no evidence of any teratogenic effect in the human although it may affect other reproductive parameters (Hall et al., 1980). Similarly, heparin is not teratogenic to rats or mice in vivo despite exposure to very high doses (Lehrer and Baker, 1974; Bertoli and Borelli, 1986).

However, when heparin was tested in vitro (Webster and Brown, 1989) it had a clear teratogenic effect, inducing exencephaly in rat embryos. The lowest teratogenic concentration was 7.5 U/ml; this concentration is greatly in excess of the normal therapeutic levels used in the treatment of deep vein thrombosis (0.3-1.0 U/ml) but is similar to that seen in patients undergoing open heart surgery or in patients who receive a bolus dose of 10,000 U (Goodman et al., 1976; Bonnar, 1976).

Do these results suggest that heparin is a potential human teratogen, and if not, why does the drug give a false positive result in vitro? A possible explanation for the teratogenic effect of heparin in vitro is that it interferes with yolk sac function, which has been postulated as the teratogenic mechanism for the acid biazo dyes (Beck et al., 1967). The rodent embryo in culture is surrounded by a yolk sac which is directly exposed to the culture serum and degrades pinocytosed protein to provide the embryo with amino acids (Beck and Lloyd, 1978). Experiments with radioactive heparin in vitro showed that the yolk sac accumulated heparin with very little reaching the embryo (Webster and Brown, 1989) but there is no evidence that this is the cause of the exencephaly. If this was the explanation for the teratogenic effect in vitro then it is unlikely that the human embryo would be at risk because, although the human embryo has a yolk sac, it is not believed to be involved in embryonic nutrition.

An alternative explanation is that the small amount of heparin which reaches the rat embryo in vitro has a direct effect on neural tube closure. It has been demonstrated that neural tube closure in rodents is dependent on the proteoglycan, heparan sulphate, and if embryos are cultured with the enzyme heparatinase neural fold elevation is inhibited (Tuckett and Morriss-Kay, 1987). Heparin is a functional analogue of heparan sulphate and is known to displace it in vitro (Cole et al., 1986); hence, it is possible that in vitro a small amount of heparin enters the embryo, either across the yolk sac or through the open mid gut, and displaces or interferes with the normal production of heparan sulphate, and prevents the normal elevation of the neural folds, resulting in exencephaly.

For both of these explanations it is unclear why heparin is not teratogenic to rodent embryos in vivo. A possible explanation is that in vivo the yolk sac and open midgut are separated from the maternal blood by Reichert's membrane, which may act as a barrier to large molecules. However, experiments with mice showed that a variety of large molecular weight compounds (horseradish peroxidase, ferritin and Thoroplast) were found inside the yolk sac after injection of these substances into a tail vein of the pregnant mother on day 7 or 8 of gestation (Poelmann and Mentink, 1982).

Potentially heparin could be teratogenic to rodent embryos in vivo, but the inability of heparin to gain access to the human embryo should prevent a similar effect in the human. Some doubt does exist as to the exact time that a true placental barrier is established in the human and whether large molecules might have access to the embryo prior to this stage.

PROPOSALS

The general theme of this paper has been that current whole animal teratology testing has serious problems with interpretation, primarily due to major differences in pharmacokinetics between species, but also because of differences in metabolic pathways between species. It is proposed that useful, additional data could be obtained by testing drugs and their metabolites on rat or mouse embryos in whole embryo culture. The drug concentrations and chemical combinations used in the testing would be determined from the pharmacokinetic and metabolic data that become available during the initial clinical trials, usually involving male volunteers. Two examples, isotretinoin and valproic acid, have shown how these data would accurately predict the teratogenicity of these compounds at therapeutic concentrations and in these instances would also predict the types of malformation. However, the possibility of false positive results with high molecular weight compounds, such as heparin, must also be considered.

Similar data can be obtained to examine the possible risk of teratogenicity from various environmental pollutants such as pesticides or heavy metals. For compounds in common usage such as pesticides, metabolic and pharmacokinetic data can be obtained from industrially exposed individuals and retrospective testing can take place. The data on Cd indicate that despite its teratogenic properties in experimental animals it is unlikely to be a human teratogen because the necessary conditions of exposure are unlikely to be fulfilled.

A variety of end points can be used in embryo culture, the most obvious being gross congenital malformations, seen as structural abnormality, and growth retardation, usually a combination of reduced somite number and reduced protein and/or DNA content. Clearly such in vitro testing does not cover all aspects of prenatal development and it is not proposed as a replacement for in vivo testing. Its major advantage is that it provides additional information that permits a more objective assessment of in vivo teratology tests.

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